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The objectives of the research program are to study the structure-immunogenicity relationships of a hypoglycosylated human tumor-specific mucin common to breast and other adenocarcinomas. Hypoglycosylation of breast mucin leads to exposure of a tumor-specific epitope (TSE). The structural and immunogenic properties of the TSE are being examined using synthetic mucin peptides and recombinant mucin proteins that contain the TSE and/or mutations in potential glycosylation sites surrounding the TSE. A single 20 amino acid tandem repeat of mucin is immunogenic. It stimulates the production of polyclonal antibodies and tumor-specific cytotoxic T lymphocytes. The single tandem repeat peptide was shown to contain few elements of preferred conformation, based on ^{1}H-NMR spectroscopy. A model of the distibution of the oligosaccharide side chains along the mucin core protein was developed showing that the carbohydrates may not surround the protein in a uniform "coating". Thus, regions of the core protein are exposed under normal circumstances, yet tumor-specific epitopes remain masked by the sugars in the non-malignant cells. Further understanding of the structure-immunogenicity relationships of tumor-specific immunogens is essential for maximizing the use of synthetic peptide immunogens and tumor-specific cells as a potential adoptive immunotherapy for patients with cancer, and the development of a potential vaccine.

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Introduction. Immunotherapy is a state-of-the-art approach to augment existing cancer therapies of radiation, chemotherapy and surgery. Immunotherapy takes advantage of the specificity of the immune system to recognize and rid the body of tumor cells. Immune cells have been identified which recognize and kill tumor cells in a specific manner [e.g. tumor-infiltrating lymphocytes (TIL); for review, 1-3], as well as in a non-specific manner [e.g. lymphokine activated killer (LAK) cells and natural killer (NK) cells]. Pertinent to this research program, T-cells (4, 5) and B-cells (6) specific for breast, ovarian and pancreatic adenocarcinomas have been described. These observations demonstrate that the body does mount an immune response against several adenocarcinomas.

Tumor proteins or tumor antigens associated with the development or expression of the malignant cell often render these cells immunogenic. Pertinent to this proposal are normal self-proteins that are aberrantly expressed or post-translationally modified on the tumor cells compared to the normal cells. Polymorphic epithelial mucin (7; mucin for this report) is a normal protein that is aberrantly expressed on the tumor cells. As such, mucin becomes a tumor-specific protein.

Mucin is the major glycoprotein of mucous secretions and is normally confined to the luminal surface of the glandular epithelial cells (7, 8). The core protein is heavily glycosylated with carbohydrate accounting for up to 80% of the glycoprotein mass (7, 8). It is the glycosylation which is responsible for the viscoelastic properties of mucus. The physicochemical properties and extracellular localization of mucin suggest that this glycoprotein has a role in surface protection and lubrication of associated tissues.

The entire mucin core protein has been cloned and sequenced (7 and references therein). To date, at least 7 different mucin proteins have been described. The hallmark of mucins are tandem repeating sequences. MUC1 is the mucin associated with breast, ovarian, pancreatic and several other adenocarcinomas. MUC1 has the 20 amino acid repeating sequence of $P^1DTRPAPGST^{10}APPAHGVTSA^{20}$ (MUC1-mucin tandem repeat; i.e., MUC1-mtr_1). Mucins are structurally polymorphic and generally contain between 40-100 tandem repeat sequences.

The structure of the MUC1-mtr_n, where n=1-3, has been partially characterized based on NMR, hydropathicity and structure prediction calculations (9-12). Viscosity measurements suggested that the structure is rod-shaped (11). Residues P^1DTRP^5 are suggested to be poly-proline β-turn elements described as "knob-like" protrusions (10-12). It is the poly-proline β-turn regions of the core protein which are suggested to be within the tumor-specific epitope (TSE) (11). This model is intriguing in that the positions of all of the potential glycosylation sites surround the TSE, which is contained in the β-turn regions. Thus, glycosylation of any, or all, of these residues may result in altered immunogenicity of the tumor-specific epitope.

The tumor-specific antigenicity and immunogenicity of mucin is inhibited by the extensive branching of the carbohydrate side chains of the mucin glycoprotein. Cancer-associated mucins are aberrantly glycosylated in that the carbohydrate side chains are shorter than those of the mucin produced by normal cells (7). These mucins have unique antigenic epitopes exposed on the core protein which are masked in the fully glycosylated form (7). Other non-tumor-specific epitopes are expressed in both the normal and tumor-associated mucins (7).

The presence of tumor-specific epitopes is evidenced by the development of a monoclonal antibody, SM3, which recognizes tumor-specific mucins and apo-mucin, but not

the fully glycosylated form (7). The epitope recognized by SM3 is the sequence P^1-P^5 in the mtr sequence (4). This epitope was identified by competition of antibody binding to tumor-specific mucin by synthetic peptides of the mtr (7).

Human cytotoxic T lymphocyte (CTL) cell lines from breast (4), ovarian (5), and pancreatic (6) adenocarcinomas have also been described which recognize mucin-expressing cell lines. One cell line, WD, was derived from the tumor-draining lymph node cells from a patient with pancreatic adenocarcinoma by continuously stimulating the cells with allogeneic pancreatic tumor cell lines. WD has a $CD3^+$, $CD4^-$, $CD8^+$ phenotype. Pancreatic mtr sequences are identical to the breast mtr (7). This explains why CTL generated against pancreatic carcinoma recognize breast carcinoma (4). The WD CTL did not recognize colon cancer cell lines, as expected, since the primary sequence of colon mucin expressed by cell lines differs from the sequence of the breast mucin peptide (7). These results show specificity of the immune recognition. Also, immunohistochemical staining and Northern blot analysis of pancreatic and breast tumor cell lines with SM3 showed that the cell lines lysed by WD CTL express the mucin epitope recognized by the monoclonal antibody (4). In similar reports, mtr peptides are recognized by breast (4) and ovarian carcinoma-specific (5) CTL, confirming the ability of adenocarcinoma-specific CTL to recognize mucin.

Fresh colon cancer tissue expressed the same mucin as breast and pancreatic cancers, i.e. MUC1 (Finn, OJ, per. commun.). The colon cancer cell lines do not express MUC1, but express other genes of the mucin family (MUC3 & MUC4). In addition, renal (Finn, OJ, per. commun.), gastric, (Metzgar, RS, per. commun.), lung and ovarian cells (7), express MUC1. This supports the hypothesis that mucin is a common tumor antigen on multiple adenocarcinomas.

Target cell recognition by these mucin-specific CTL lines is Human Leukocyte Antigen (HLA)-non-restricted. This was shown by the ability of mucin-specific CTL cell lines to recognize and kill non-HLA-matched tumor target cell lines. One explanation for this is that mucin, due to its polymorphic nature (i.e., mucin contains 40-100 tandem repeat sequences), is a multivalent antigen. Multiple epitopes on the tumor cell surface not associated with HLA are postulated to bind to, and cross-link, the T cell receptor (TCR), thus leading to CTL activation and target killing (4, 13, 14). Another explanation for the lack of HLA-restriction is that adenocarcinoma cells do not express HLA-class I molecules (15). By contrast, CTL recognition of target cells bearing a single tandem repeat of mucin are HLA-restricted (16).

Multiple approaches have been used to generate mucin tumor-specific CTL. These methods have involved the use of hypoglycosylated mucin, obtained from autologous and allogeneic tumor cells or expressed as a recombinant protein, as the immunogen. For example, mucin has been expressed in a mammalian system using phenyl-N-acetyl-α-galactosaminide (17), an inhibitor of glycosyl transferases involved in the early chain elongation of O-linked oligosaccharides. However, the initial N-acetylgalactosamine (GalNAc) still modifies the core protein (17). Personal communications (18, 19) have stated that SM3 binds to MUC1 modified by a single NAcGal, but no published evidence supports this hypothesis. Also, Finn and co-workers (20) have used a truncated mucin, suggested to be non-glycosylated using antibodies claimed to recognize glycosylated vs. non-glycosylated mucin; however, these mucins were not biochemically proven to be non-glycosylated. Therefore, this raises questions of the utility of this approach for generating mucin-specific

CTL. We address the issue of obtaining reproducibly, non-glycosylated mucin immunogens below and the structure-immunogenicity relationship of tumor-associated MUC1.

Body. Antigenicity and immunogenicity of synthetic and recombinant mucin peptides. To compare the ability of different immunogens to stimulate and expand tumor-mucin-specific CTL, non-glycosylated mucin peptides have been synthesized. The synthetic nature of these peptides ensures reproducible non-glycosylation of the immunogen and unmasking of core protein tumor-specific epitopes. The synthetic peptide immunogens are the native MUC1-mtr$_n$ (P^1DTRPAPGST^{10}APPAHGVTSA^{20}), where n=1-3, and the mutated peptides (T3N)-MUC1-mtr$_1$ and (T[10, 18]N)-MUC1-mtr$_1$.

Fig. 1 shows that both the native and mutated mucin MUC1 peptides are recognized by mucin-specific polyclonal antiserum. Fig. 2 shows that the native and mutated mucin

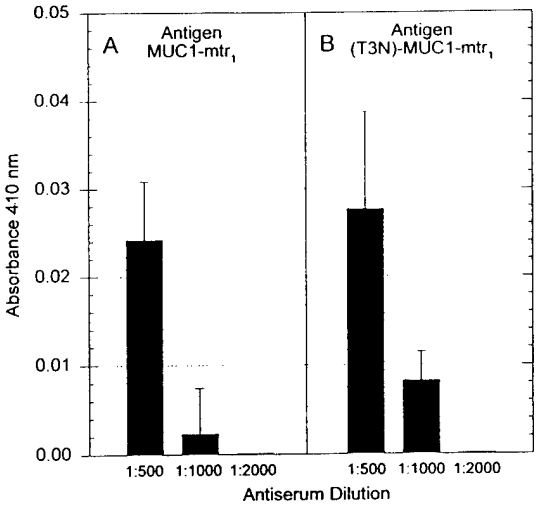

Figure 1. Cross-reactivity of (A) native MUC1-mtr$_1$ and (B) mutated (T3N)-MUC1-mtr$_1$. Cross-reactivity was determined by ELISA with anti-mucin polyclonal antiserum (21). The peptides were probed with serial dilutions of anti-serum. The mucin peptide-anti-mucin complexes were quantified by reaction with anti-rabbit IgG-horse radish peroxidase conjugate and reading the absorbance of the chromogenic substrate at 410 nm.

peptides are immunogenic and can elicit an antibody response. Furthermore, antisera raised against the native peptide cross-reacted with the mutant peptide, and vice versa, with similar titers as against their antigen (not shown). Thus, mutation of the MUC1 peptide did not alter its immunogenicity.

A synthetic gene encoding five tandem repeats of the tumor-specific epitope of human mucin was designed for efficient cloning and expression in *E. coli*. The synthetic gene was cloned in the correct reading frame into the MBP-fusion expression vector pMAL-p2. Bacterial clones containing the mucin synthetic gene insert were shown to produce a protein with characteristics consistent with the intended recombinant fusion protein, MBP-mtr$_5$. Upon addition of IPTG to induce production of the fusion protein, the synthesis of a protein of expected molecular weight that constitutes a substantial percentage of the total protein is detectable on Coomassie-brilliant blue-stained SDS-PAGE gels. The inducible protein binds

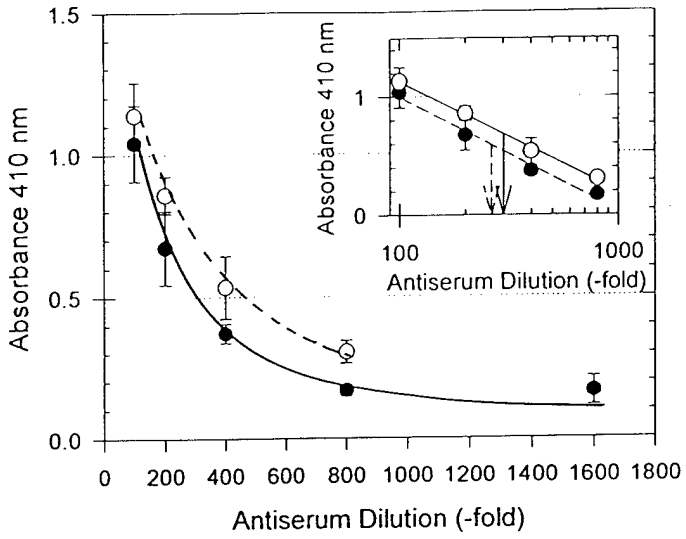

Figure 2. Titer of native and mutated MUC1 polyclonal antisera. Antibody titers were determined by ELISA. The titers of the antisera were determined as the 50% point. (●) MUC1-mtr_1 assayed against anti-MUC1-mtr_1 and (○) (T3N)-MUC1-mtr_1 assayed against anti-(T3N)-MUC1-mtr_1. *Inset*: Semi-log plot of cross-reactivity of antisera with the respective antigen.

to amylose resin, verifying the presence of the MBP moiety of the fusion protein, and is reactive with monoclonal antibodies which are specific for human mucin, verifying the presence of the mtr_5 polypeptide. Furthermore, the fusion protein produced represents a significant fraction of the cellular protein, and is not heavily degraded. A substantial portion of the MBP-mtr_5 fusion is secreted into the periplasm, a characteristic which will greatly facilitate purification of the protein. Preliminary experiments indicate that purification of the fusion protein from the periplasm as a first step, followed by amylose resin chromatography will produce an MBP-mtr_5 protein preparation of high purity (data not shown). The non-glycosylated MBP-MUC1-mtr_5 (Dolby, N., Wright, S.E., Dombrowski, K.E, manuscript in preparation) is recognized by tumor-specific antibodies and contains the tumor-specific epitope.

Mass spectrometric analysis of synthetic mucin tandem repeat peptides. Synthetic MUC1-mtr_1, (T3N)-MUC1-mtr_1 and [T(10, 18)]-MUC1-mtr_1 peptides were characterized by electrospray ionization mass spectrometry (ESMS). The peptides were found to have the appropriate molecular weights and to be of high quality giving no mass spectrometric evidence of the presence of blocking groups or contamination by other peptides. The mass spectra were dominated by multiply protonated species but all exhibited adduction with other cationic species such as sodium, potassium and iron (Fig. 3). These cationic contaminants are common to ESMS and result from contact with glass and the stainless steel in the ion source of the mass spectrometer. The mass spectra of all the peptides studied gave evidence of M-18 amu. This is presumably due to dehydration of S or T during the ESMS analysis.

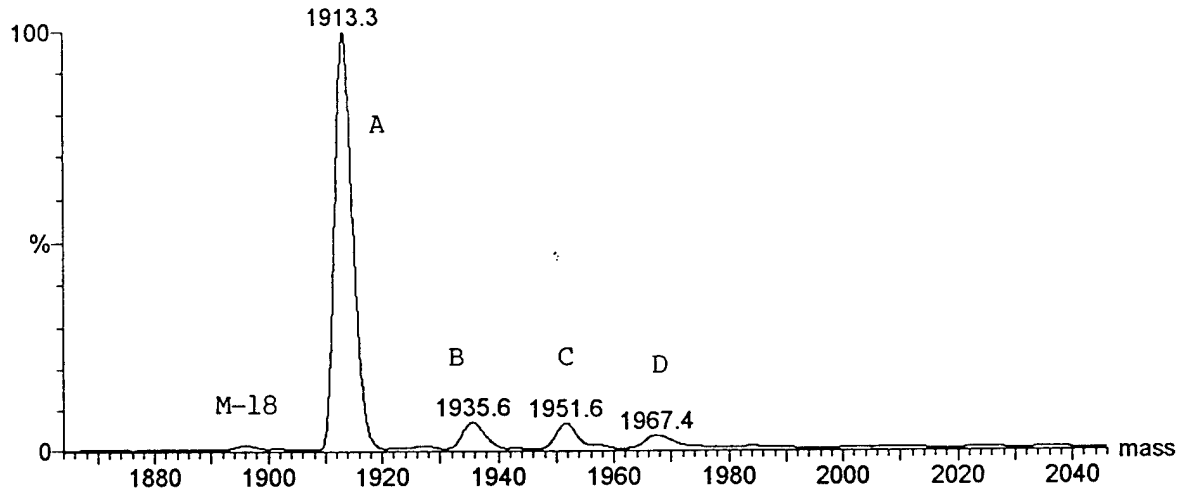

Figure 3. Transformation of m/z data to a molecular mass scale: peak A is the (T3N)-MUC1-mtr$_1$; peak B is the sodiated adduct; peak C is the potassiated adduct; peak D is the iron adduct.

Table 1. Molecular weights of synthetic peptides.

Sequence	Name	Measured	Theoretical Monoisotopic[1]	Mol. Wt. Average
PDNRPAPGSTAPPAHGVTSA	(T3N)MUC1-mtr$_1$	1899.1	1898.92	1900.04
PDTRPAPGSTAPPAHGVTSA-PDTRPAPGSTAPPAHGVTSA	MUC1-mtr$_2$	3755.5	3753.06	3756.06
PDTRPAPGSNAPPAHGVNSA	(T[10, 18]N)-MUC1-mtr$_1$	1911.8	1911.92	1913.04

[1]Molecular weights below 2500 amu are determined from the doubly protonated ion, [M+2H]2+/2, and should be compared to the theoretical monoisotopic mass.

Development of tumor-specific CTL lines. We have developed mucin specific CTL from peripheral blood mononuclear cells (PBMC) and tumor-infiltrating lymphocytes (TIL) from breast and ovarian cancer patients to study the immunogenicity of tumor-specific mucins. The synthetic non-glycosylated MUC1-mtr$_1$ and (T3N)-MUC1-mtr$_1$ peptides elicit a mucin specific response from both PBMC and TIL cells. Specifically, PBMC from a breast cancer patient, and TIL from both breast and ovarian tumor samples were cultured in the presence of either anti-CD3 (OKT3) + IL-2 (recombinant human interleukin 2), MUC1-mtr$_1$ + IL-2 or (T3N)-MUC1-mtr$_1$ + IL-2. We analyzed the expansion, phenotype (including TCR Vß repertoire), and cytotoxicity of these activated cells following restimulation. (*Note*: All cultures contain IL-2, whether or not it is specifically stated.)

The expansion of CTL from the PBMC of a breast cancer patient using the mucin peptides was comparable to the expansion of CTL by anti-CD3 cross-linking (not shown). The PBMC cultured with MUC1-mtr$_1$ + IL-2 or (T3N)-MUC1-mtr$_1$ + IL-2 had similar phenotype profiles with respect to CD4$^+$ (40%), CD8$^+$ (30%), and CD28$^+$ (54-59%) subpopulations. The anti-CD3 + IL-2-stimulated T cell population had a CD4$^+$ (49%), CD8$^+$ (50%) distribution, and a slightly higher level of CD28$^+$ (79%) cells. The anti-CD3-stimulated T cell cultures treated with soluble anti-CD28 monoclonal antibody displayed the highest level of CD28$^+$ cells (94%). The phenotype of the peptide stimulated cultures also showed higher levels of CD8$^+$/CD56$^+$ double positive T cells as compared to the anti-CD3 activated cells. This demonstrates that the bulk population of these cells is not natural killer (NK) cells.

The proliferative response was measured by ^{3}H-thymidine incorporation (Fig. 4). Proliferation induced by (T3N)-MUC1-mtr$_1$ following a 17-day stimulation was 3-fold higher than that resulting from stimulation by either MUC1-mtr$_1$ or anti-CD3 activated T cells. These results show a mucin-specific response and that a mutation in a potential glycosylation site enhanced the proliferative response of these T cells.

The cytotoxic response against the breast cancer cell line, MCF7, that expresses hypoglycosylated mucin, showed that mucin peptide-stimulated PBMC cultures had higher specific target cell lysis against the mucin-expressing target cells, as compared with anti-CD3

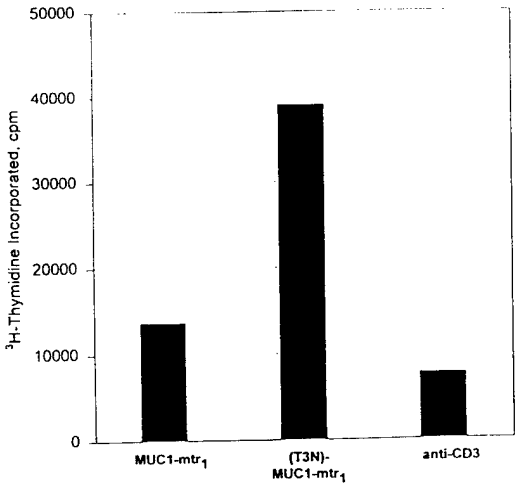

Figure 4. Proliferative response after peptide stimulation. PBMC from a breast cancer patient were cultured with anti-CD3 (OKT3) + 100 IU/ml IL-2, MUC1-mtr$_1$ + 100 IU/ml IL-2 or (T3N)-MUC1-mtr$_1$ + 100 IU/ml IL-2 for 14 days. Proliferation was measured by the incorporation of ^{3}H-thymidine on day 17.

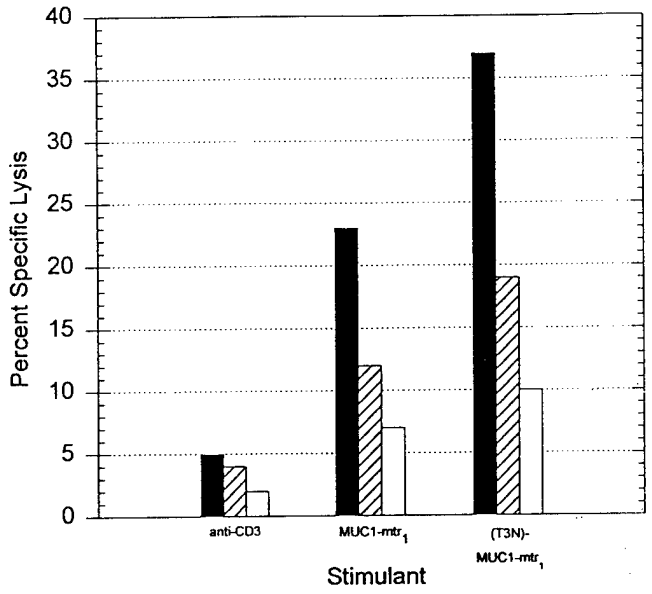

Figure 5. Cytotoxic response after peptide restimulation. PBMC were cultured as described in Fig. 4. The cultures were restimulated with either the respective mucin peptide + IL2 or anti-CD3 + IL2 after 14 days in culture. The cytolytic activity of the cultures was assayed 5 days after restimulation against MCF-7 tumor target cells by measuring the percent specific ^{51}Cr-release. Effector-target cell ratios assayed were 20:1 (*dark bars*); 10:1 (*dashed bars*); 5:1 (*open bars*).

stimulated cultures (Fig. 5). These results demonstrate that a significant population of the mucin-stimulated cultures are tumor-specific cytolytic cells. If lymphokine activated killer (LAK) cells were the dominant population in these cultures, the expected cytolytic activity would have been comparable between anti-CD3 stimulated cultures and mucin stimulated cultures. Since this was not the case, the data show that mucin-specific cytolytic cells are being expanded. Also, HLA-typing demonstrated that this patient and MCF-7 were not matched. Therefore, this suggests that the cytolytic activity of these cultures is HLA-non-restricted.

TCR analysis of these cultures indicated that each immunogen induced expansion of a narrowed T cell population with each population expressing a unique Vß repertoire. Anti-CD3 activated cultures showed polyclonal expansion of T cells with Vß 1, 4 and 13 repertoire (above an arbitrary background set at 5%). MUC1-mtr$_1$ stimulated cells showed predominantly Vß 2, 13, 14, 18 and 24 positive populations. By contrast, Vß 4, 6, 7, 13 and 18 positive cells were predominantly expanded in (T3N)-MUC1-mtr$_1$ stimulated cultures (Fig. 6).

Two of two experiments performed with PBMC from this breast cancer patient yielded similar results. Thus, these results indicate that our approach to the stimulation and expansion of tumor mucin-specific CTL is reproducible at the single patient level.

We have performed additional experiments examining the expansion of tumor-specific CTL from breast cancer TIL. Briefly, TIL were cultured with 500 IU/ml interleukin 2 (IL-2) for three weeks in the presence of autologous tumor. IL-2 was then reduced to 130 IU/ml $\pm$ 1 μg/ml MBP-MUC1-mtr$_7$ (17). Cells <u>not</u> stimulated with recombinant mucin protein showed significant killing of both target cell lines demonstrating NK/LAK activity (Fig. 7). However, mucin stimulated cultures did not lyse these target cells, demonstrating that NK/LAK activity is not present. Further characterization of this cell line is in progress.

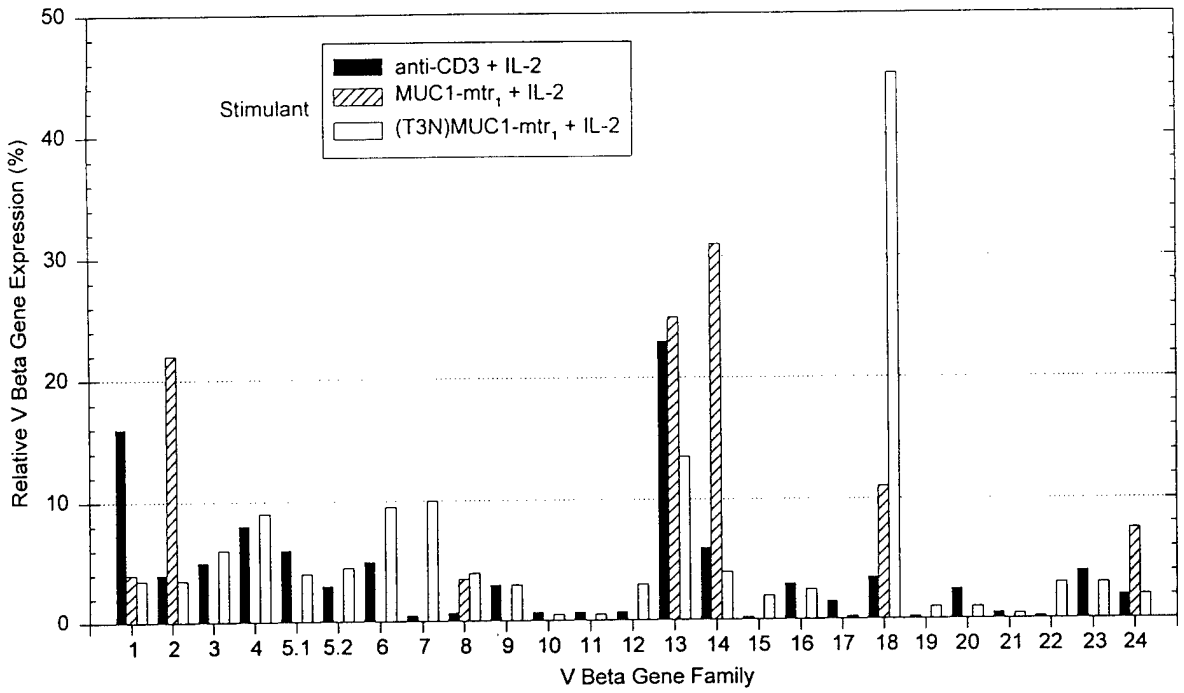

Figure 6. TCR analysis of mucin peptide stimulated PBMC cultures from a breast cancer patient. PBMCs were stimulated for 14 days and then restimulated for 3 days with indicated stimulants as described in Fig. 4. Vβ gene expression was then determined by PCR of RNA of the cells.

These experiments have been repeated by stimulating PBMC from a third patient with breast cancer with the mucin immunogens. Fig. 8 shows that these cytolytic cells are also specific for MCF-7 tumor cells. Although the specific lysis is low, the CTL are tumor-specific and lack NK/LAK activity since the NK/LAK target cells K562 and Raji, respectively, are not lysed. The reason for the low cytolytic activity is not yet understood. This experiment confirms the above results (Fig. 7) in that mucin stimulation of human lymphocytes from patients with breast cancer results in the generation of tumor-specific CTL and not NK/LAK activity.

Mucin specific CTL were also generated from an ovarian cancer TIL sample using culture conditions slightly different than those used for breast cancer PBMC and TIL. Ovarian cancer TIL were cultured either in the presence of IL-2 alone, MUC1-mtr$_1$ + IL-2, or (T3N)-MUC1-mtr$_1$ + IL-2 for 29 days and analyzed for the phenotype changes, cytolytic activity and TCR repertoire. All three culture conditions had predominant CD4$^+$ (helper) and CD45RO$^+$ (memory) T cells. The anti-CD3-, and (T3N)-MUC1-mtr$_1$-stimulated TIL showed a slight increase in CD8$^+$ T cells as compared to the MUC1-mtr$_1$ cultured T cells. However, there was a clear difference in the cytotoxicity of these cells when tested against MCF7 target cells.

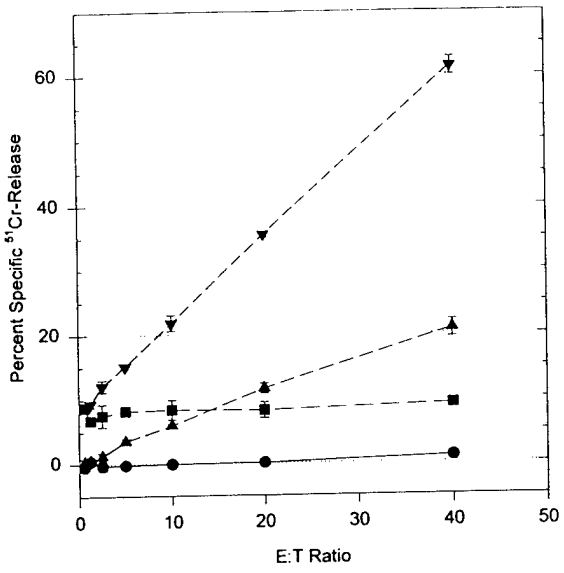

Figure 7. Cytolytic activity of mucin-stimulated breast cancer TIL against NK/LAK target cells. TIL were cultured with 500 IU/ml IL-2 for three weeks in the presence of autologous tumor. IL-2 was then reduced to 130 IU/ml ± 1 μg/ml MBP-MUC1-mtr$_7$ fusion protein (21). ^{51}Cr-release assay of these effector cells against K562 (NK target) and Raji (LAK target) cells was performed as described previously (4, 5). (●) TIL stimulated with 1 μg/ml MBP-MUC1-mtr$_7$ and assayed against Raji cells; (▲) TIL stimulated without MBP-MUC1-mtr$_7$ and assayed against Raji cells; (■) TIL stimulated with 1 μg/ml MBP-MUC1-mtr$_7$ and assayed against K562 cells; (▼) TIL stimulated without MBP-MUC1-mtr$_7$ and assayed against K562 cells. Bars are standard error.

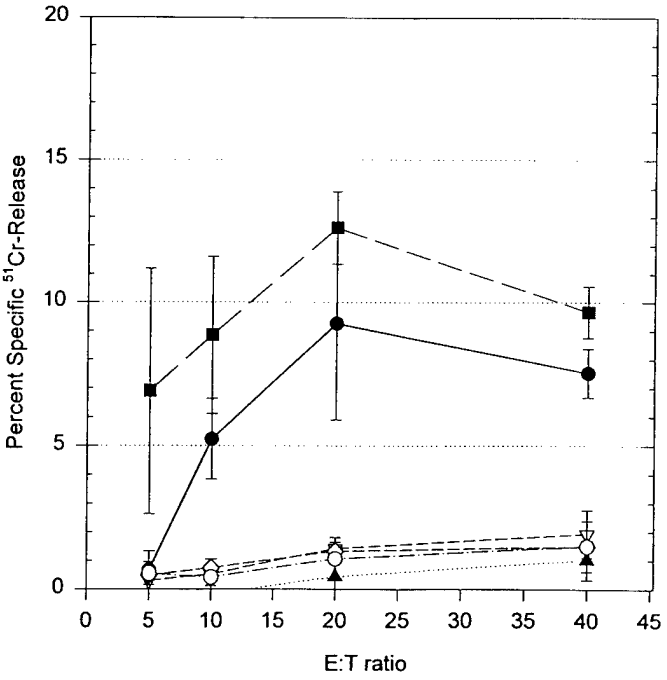

Figure 8. Target cell specificity of breast cancer CTL. CTL were generated by culturing lymph node cells with 100 IU/ml IL-2 for 30 days + 1 μg/ml of the mucin peptide indicated below. Culture media were changed twice a week. Cytolytic activity was determined by ^{51}Cr-release assay (4, 5). (●)MUC1-mtr$_1$-stimulated and assayed against MCF7 cells; (■) (T3N)-MUC1-mtr$_1$-stimulated and assayed against MCF7 cells; (▲) MUC1-mtr$_1$-stimulated and assayed against K562 cells; (▽) (T3N)-MUC1-mtr$_1$-stimulated and assayed against K562 cells; (◇) MUC1-mtr$_1$-stimulated and assayed against Raji cells; (○) (T3N)-MUC1-mtr$_1$ stimulated and assayed against Raji cells. Bars are standard error.

The cultures expanded with IL-2 alone did not show significant cytotoxicity against MCF7 target cells (~ 2 %), whereas cultures stimulated with either mucin peptide showed significant killing activity (Fig. 9). MUC1-mtr$_1$-stimulated cultures demonstrated moderate killing (~ 8%); a significantly higher target cell lysis (~ 25%) was observed when (T3N)-MUC1-mtr$_1$-stimulated cells were used as effectors (Fig. 9). Thus, mucin peptide stimulation also expands the cytolytic activity of ovarian TIL.

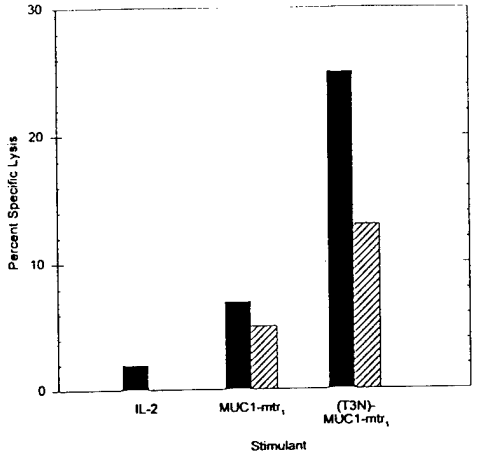

Figure 9. Cytotoxic activity of ovarian TIL after mucin peptide stimulation. Cytotoxic activity of ovarian TIL cultured with IL-2 alone and mucin peptides + IL-2 against MCF7 target cells by ^{51}Cr release assay (4, 5). Effector-target cell ratios assayed were 20:1 (*dark bars*); 10:1 (*dashed bars*).

TCR analysis of the cultures expanded from the ovarian cancer TIL showed different Vβ repertoires in both the MUC1-mtr$_1$ and the (T3N)-MUC1-mtr$_1$ stimulated cultures (Fig. 10). TIL cultured with IL-2 alone showed predominant Vβ 13.2 and 22.1 populations. MUC1-mtr$_1$ stimulated TIL had a dominant Vß 5.4 and 18.1 populations, whereas (T3N)MUC1-mtr$_1$ stimulated cultures showed Vß 1.1, 2.1b, 3.1 and 5.2 as the predominant populations. Thus, these results also show oligoclonal expansion of a limited Vβ repertoire following mucin peptide stimulation. This is similar, at least in part, to the work by Peoples and co-workers showing expansion of Vβ 2 for ovarian TIL (22). Functional correlation of the TCR Vβ usage and response against the autologous tumor are in progress.

HLA class I-bound mucin peptide. Barnd et al. (4) and Ioannides et al. (5) postulated that the mucin epitope bound to HLA class I (T-cell epitope) is included in, or adjacent to, the same epitope that is recognized by the SM3 monoclonal antibody (7). This was based on the observation that SM3 bound to cancer cells expressing mucin inhibited the recognition and lysis of the target cell by CTL. The CTL epitope was narrowed to residues P^1-T^{10} which includes the sequence recognized by SM3 (5). Antibodies to sequences outside these 10 amino acids had no inhibitory effect on the lysis of mucin-bearing adenocarcinomas by CTL (7). This suggests that the monoclonal antibody binding site and the T-cell epitope are the same, or near one another. However, it was not clear whether the inhibition of cytolytic activity was due to steric hindrance as a result of antibody binding. The exact sequence of the HLA-bound tumor-specific mucin T cell epitope remains to be determined.

We have used an algorithm to predict the potential HLA class I-bound mucin peptide (Table 2). The algorithm was developed for peptides bound to HLA A-2 (23). This approach assigns a value of +3 for strong binding residues, +2 for moderate binding

A. MUC1-mtr$_1$

Score	P	D	T	R	P	A	P	G	S	T	A	P	P	A	H	G	V	T	S	A	P	D	T	R	P	A	P	G
0	P	D	T	R	P	A	P	G	S																			
2		D	T	R	P	A	P	G	S	T																		
1			T	R	P	A	P	G	S	T	A																	
3				R	P	A	P	G	S	T	A	P																
4					P	A	P	G	S	T	A	P	P															
0						A	P	G	S	T	A	P	P	A														
2							P	G	S	T	A	P	P	A	H													
1								G	S	T	A	P	P	A	H	G												
6									*S*	*T*	*A*	*P*	*P*	*A*	*H*	*G*	*V*											
2										T	A	P	P	A	H	G	V	T										
2											A	P	P	A	H	G	V	T	S									
5												P	P	A	H	G	V	T	S	A								
3													P	A	H	G	V	T	S	A	P							
1														A	H	G	V	T	S	A	P	D						
1															H	G	V	T	S	A	P	D	T					
0																G	V	T	S	A	P	D	T	R				
1																	V	T	S	A	P	D	T	R	P			
3																		T	S	A	P	D	T	R	P	A		
2																			S	A	P	D	T	R	P	A	P	
2																				A	P	D	T	R	P	A	P	G

B. (T3N)-MUC1-mtr$_1$

Score	P	D	N	R	P	A	P	G	S	T	A	P	P	A	H	G	V	T	S	A	P	D	N	R	P	A	P	G
0	P	D	N	R	P	A	P	G	S																			
2		D	N	R	P	A	P	G	S	T																		
1			N	R	P	A	P	G	S	T	A																	
3				R	P	A	P	G	S	T	A	P																
4					P	A	P	G	S	T	A	P	P															
0						A	P	G	S	T	A	P	P	A														
2							P	G	S	T	A	P	P	A	H													
1								G	S	T	A	P	P	A	H	G												
6									*S*	*T*	*A*	*P*	*P*	*A*	*H*	*G*	*V*											
2										T	A	P	P	A	H	G	V	T										
2											A	P	P	A	H	G	V	T	S									
5												P	P	A	H	G	V	T	S	A								
3													P	A	H	G	V	T	S	A	P							
1														A	H	G	V	T	S	A	P	D						
1															H	G	V	T	S	A	P	D	N					
0																G	V	T	S	A	P	D	N	R				
3																	V	T	S	A	P	D	N	R	P			
2																		T	S	A	P	D	N	R	P	A		
3																			S	A	P	D	N	R	P	A	P	
1																				A	P	D	N	R	P	A	P	G

Table 2. Prediction of the potential HLA-bound mucin peptide of the (A) MUC1-mtr$_1$ and (B) the mutated (T3N)-MUC1-mtr$_1$. Bolded is the 20 amino acid mucin peptide. The nonamer peptide italicized and underlined is the dominant predicted T cell epitope.

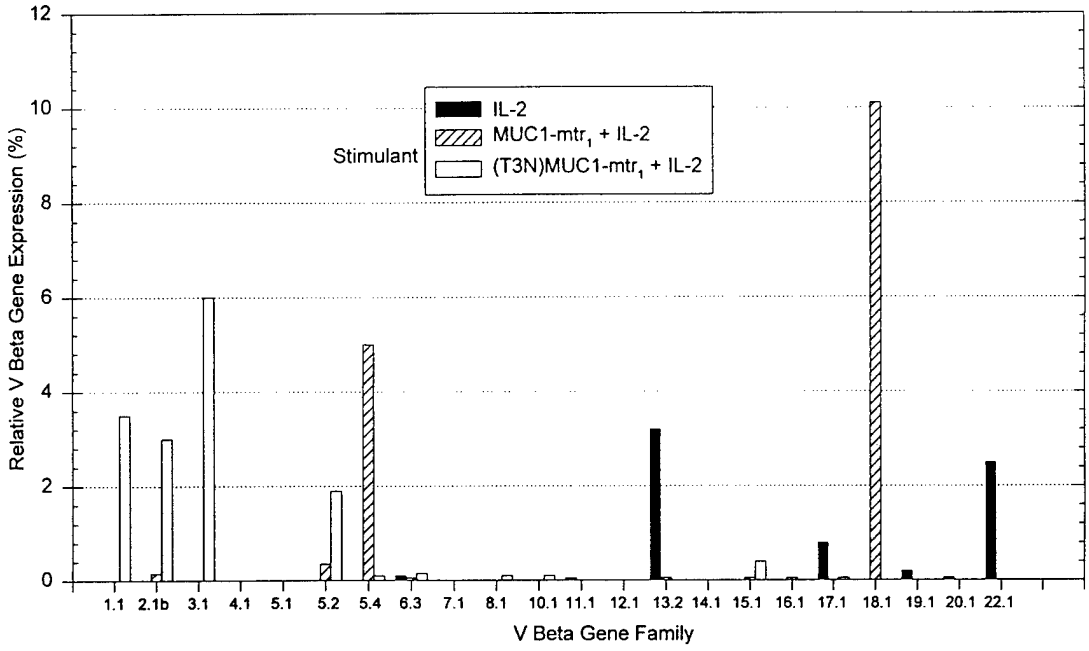

Figure 10. TCR analysis of mucin peptide stimulated ovarian cancer TIL. TCR analysis of ovarian cancer TIL cultured with IL-2 alone or mucin peptides + IL-2 cultured for 29 days was performed using an RNase protection assay.

residues, +1 for weak binding, and 0 for not found. Table 2 shows for both the MUC1-mtr_1 and (T3N)-MUC1-mtr_1 the nonamer $S^9TAPPAHGV^{17}$ has the highest predictive value for the potential T cell epitope. This suggests that a mutation in a potential glycosylation site within the monoclonal antibody tumor-specific epitope is not within the potential T cell epitope. Evidence has also been published suggesting that this nonamer may be a T-cell epitope (24). This peptide bound to HLA-A1, -A2.1, -A.3 and -A11. The affinity of HLA-A11 for this peptide was close to that found for HLA-bound non-mucin peptides. Since this peptide is "outside" the range of the T cell epitope suggested by Ioannides et al. (5), it is reasonable to conclude that the inhibition of cytolytic activity by SM3 was due to steric hindrance and not to masking of the epitope by the antibody. These results also suggest an HLA class I-restricted T cell antigen recognition of tumor-specific mucin.

Conformation of MUC1-mtr_1 peptides. We are also studying the conformational preferences of human mucin (Ribeiro, AA, Spicer, LD, Wright, SE & Dombrowski, KE, manuscript in preparation). We have made the sequential proton assignments for the MUC1-mtr_1 (Fig. 11A) and (T3N)-MUC1-mtr_1 (Fig. 11B) in water based on COSY, TOCSY and NOESY ^{1}H-NMR spectroscopy. We have observed reasonable dispersion of backbone NH and α-CH resonances.

The temperature dependence of the backbone NH resonances was also examined over the range of 0-35°C. Temperature coefficients are of intermediate value and show little evidence for a single stable secondary structure (not shown). However, three residues (R^4,

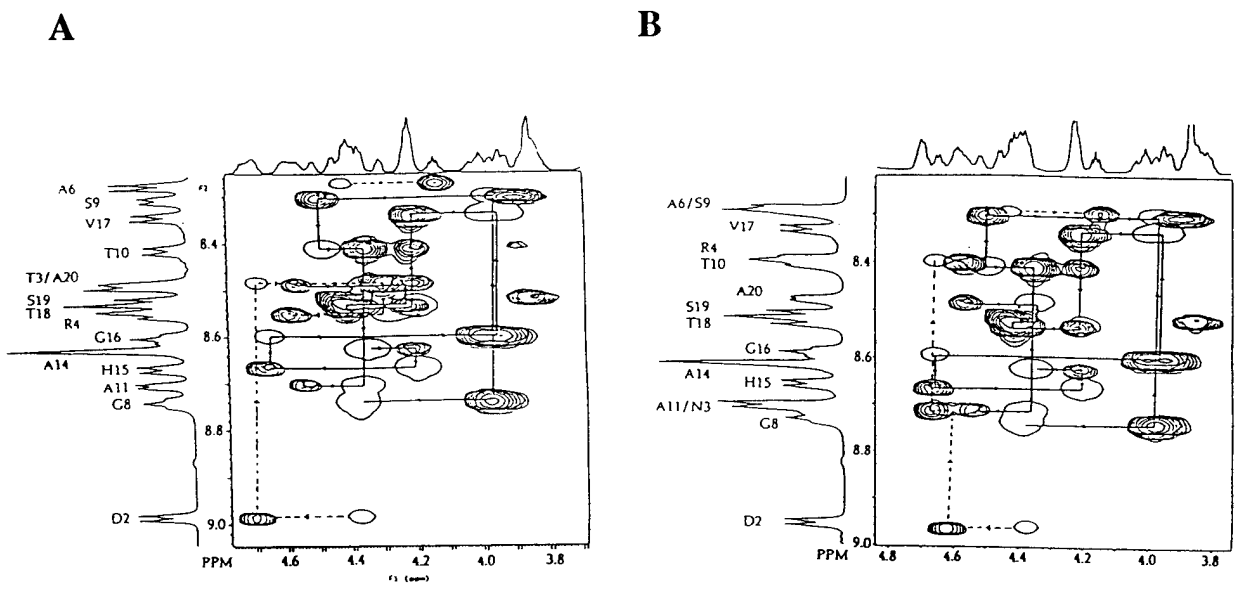

Figure 11. ^{1}H-NMR network for backbone assignments of (A) MUC1-mtr$_1$ and (B) (T3N)-MUC1-mtr$_1$ illustrated on overlaid plots of TOCSY and NOESY spectra.

S^9, and G^{16}) show less temperature dependence. This suggests that these residues remain in a more stable conformation relative to the other amino acids.

Based on NMR studies in d^6-DMSO (12) a β-turn was proposed as a major conformational feature of native mucin peptide. In initial NMR studies in aqueous phosphate buffer at pH 6.8 (10), the single native mucin peptide yielded broad, overlapped NH signals, and these workers concluded that the native repeat peptide is largely disordered in solution. More recently, better resolved 2D spectra (11) also indicate a largely unordered structure with evidence for "knob-like" domains.

Our data obtained at pH 4 show good dispersion of backbone NH and α-CH resonances in both the native and mutant peptides. This supports the case for the presence of elements of secondary structure. Our data also provide clear evidence that no large global changes occur upon substitution of the uncharged polar Thr side chain [-CHOH-CH_3] with the uncharged polar Asn side chain [-CH_2-$CONH_2$]. Only local shift changes are seen: the T^3 NH is replaced by the N^3 NH and concomitantly the R^4 NH moves upfield. The remaining amino acid resonances have similar shifts and similar temperature behavior. Since there is little evidence for a single stable secondary structure, it may well be that more than one tandem repeat sequence is needed to stabilize a more ordered polypeptide. However, a sufficient population of preferred peptide conformers may exist in a single tandem repeat to elicit the immunogenic response.

While our detailed NMR efforts have focussed on the single 20 amino acid tandem repeat peptide, MUC1-mtr$_1$, we have recently obtained our first NMR observations for the 40 amino acid, double tandem repeat peptide, MUC1-mtr$_2$. The His^{15} and His^{35} C2H proton signals are found to be equivalent in chemical shift in MUC1-mtr$_2$, and to resonate at the

same value as the His^{15} in MUC1-mtr_1. Since these protons are not exchangeable with solvent water protons, we can compare the intensities of the NH signals of MUC1-mtr_1 and MUC1-mtr_2 by scaling relative to the number of histidine protons. Fig. 12 shows the NH resonances at 5 and 35°C, respectively. The bulk of the NH resonances at both temperatures are found to behave similarly to the His C2H signals, i.e., the shifts of the second repeat are coincident with the shifts of the first repeat unit. However, a closer examination of the 5°C

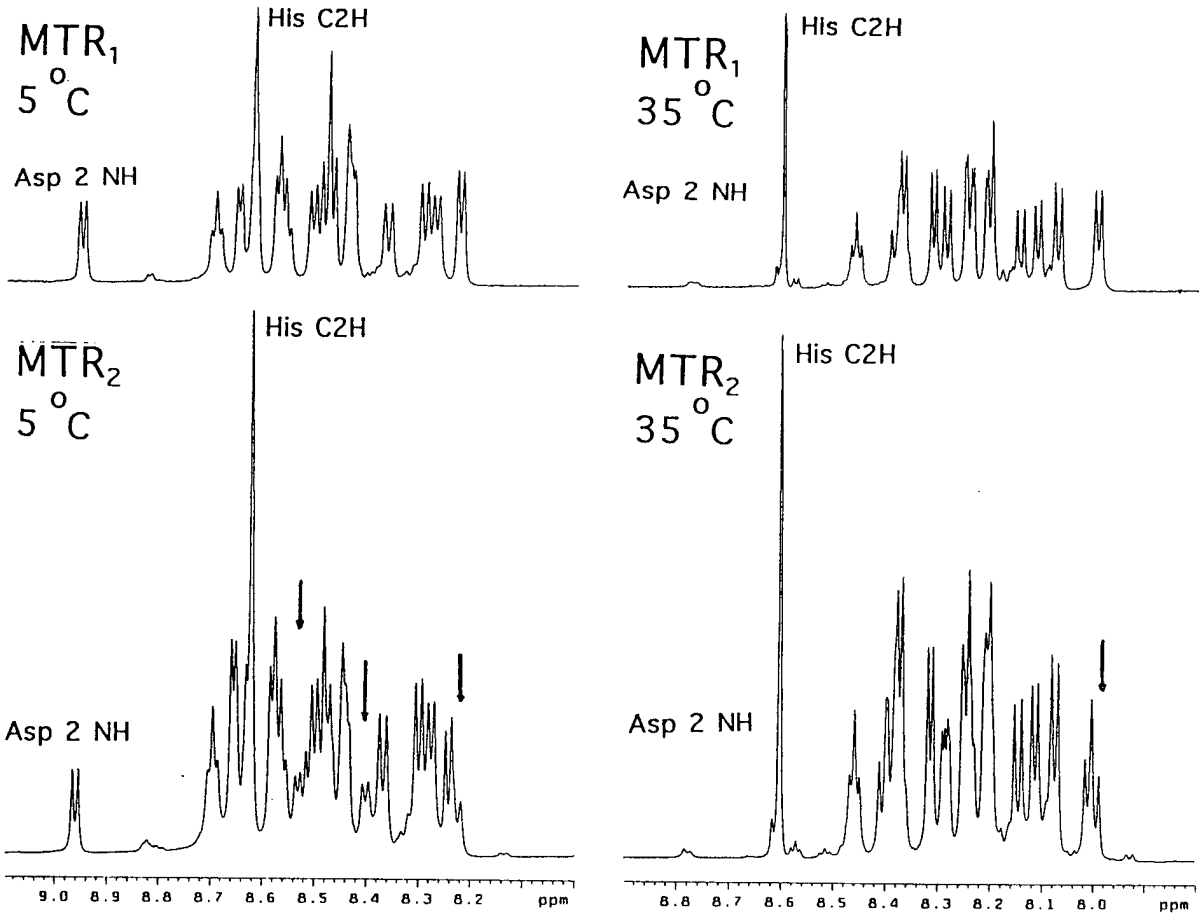

Figure 12. Comparison of the amide NH region of MUC1-mtr_1 (top panel) and MUC1-mtr_2 (bottom panel). NMR spectra were obtained in water at 5 and 35°C.

spectra reveals three new NH resonances in MUC1-mtr$_2$ not observed in MUC1-mtr$_1$, and that the resolved Asp^2 NH signal 8.96 ppm integrates to only one proton in both the single and double tandem repeat peptides. This implies that Asp^2 and Asp^{22} of MUC1-mtr$_2$ are not coincident in chemical shift. Moreover, at 35°C, loss of NH intensity due to exchange with solvent is apparent mainly at the Asp^2 NH in both mucin peptides. MUC1-mtr$_2$ has not previously been studied at this detailed level, and further work should provide new insights into the conformational preferences of these tandem repeats.

Surface Analysis of Native and Deglycosylated Mucin. X-ray photoelectron spectroscopy (XPS) is a surface sensitive analytical technique which measures the binding energy of electrons in atoms and molecules. The binding energy can be related to the molecular bonding or oxidation state of an element in the outermost layer of a material (< 100Å). Thus, XPS is able to identify chemical species present on the surface of a molecule. The quantitative XPS results of C, O and N for amino acids, simple carbohydrates and peptides of human mucin have been previously determined (25).

One of the goals of the proposed research is to correlate the amount of carbohydrate coating (e.g., thickness) with immunological response for human mucin. Mucin from human breast milk was chosen to model the fully glycosylated mucin. The breast milk mucin was purified according to published procedures (26). Based on sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the mucin sample was homogeneous and free from low molecular weight contaminating proteins. Direct analysis of the molecular mass of the mucin purified from human breast milk by mass spectrometry have not been successful to date, most likely due to the large mass (> 10^6 daltons) which is beyond the technology to measure at present.

Fig. 13 shows the overall XPS spectrum for the fully glycosylated native breast milk mucin. The three major atomic constituents of the mucin were observed in this spectrum: C, N and O. Since the XPS technique is surface sensitive and the mucin oligosaccharide side chain contains a low abundance of N (7, 25), a low relative atomic percent composition of N photoelectrons (~ 0.3%; 26) would be expected if the carbohydrate completely covers the core protein, whereas a fully deglycosylated mucin core protein would exhibit an atomic percent composition of ~ 15-17% (25, 26). If a random or globular arrangement of the oligosaccharide side chain exists on the core protein, or if the side chain is uniformly thin, a N 1s signal intensity of > 0.3 atomic %, but < 15-17 atomic % would be expected. Quantitation of the XPS spectrum for the fully glycosylated breast milk mucin showed atomic percent compositions for C of 75.9%, 3.5% for N and 19.4% for O. Thus, these results show that there is a greater than expected contribution of N 1s photoelectrons to the XPS spectrum. This is most likely due to the contribution of the N of the protein backbone. This result implies that the carbohydrate is uniformly thin (< 100 Å) (Fig. 14A) or does not completely "coat" the core protein in the native mucin molecule (Fig. 14B).

Conclusions. The goal of this work is to understand the structure-immunogenicity relationships of the tumor-specific mucin. This information will be useful in developing immunogens for the adoptive immunotherapy of adenocarcinomas and as potential vaccines.

It has been demonstrated in the first two years of this grant that a conservative mutation of a potential glycosylation site in a tumor-specific epitope is effective in eliciting both humoral and cellular immunogenic responses that are tumor-specific. These data demonstrate that synthetic, non-glycosylated mucin peptides serve as immunogens to

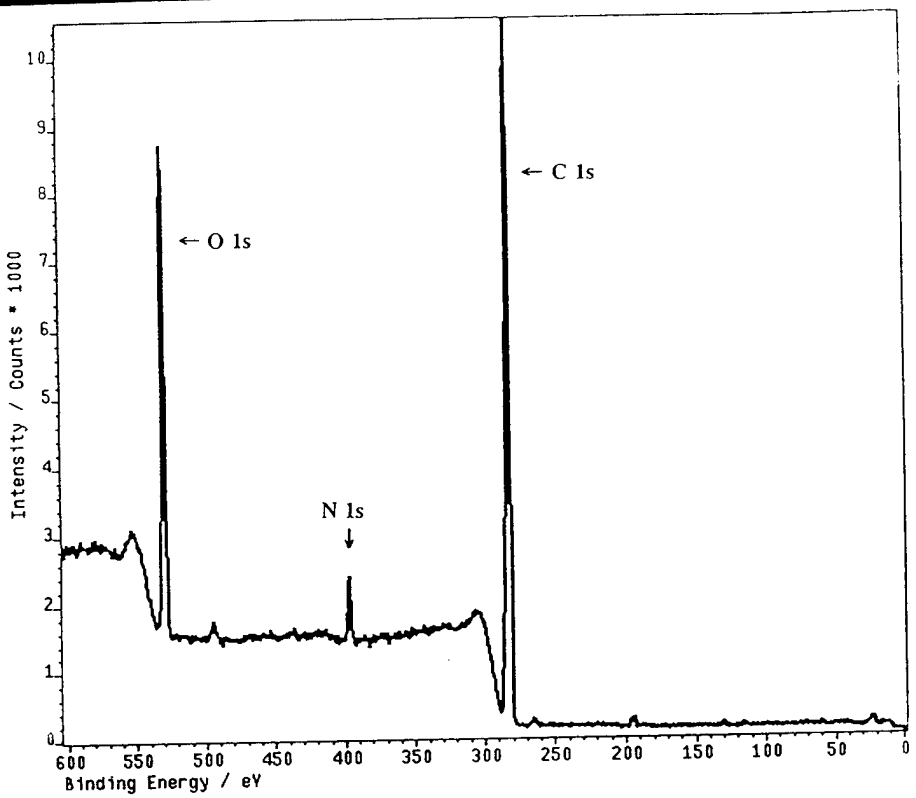

Figure 13. Overall XPS photoelectron spectrum of purified human breast milk mucin. Lyophilized purified breast milk mucin was analyzed in powder form on a Kratos Axis x-ray photoelectron spectrometer (25).

reproducibly generate tumor-specific CTL from PBMC, TIL and lymph node cells from patients with adenocarcinomas. The expansion of mucin-specific T cells is oligoclonal. Antigen recognition at the level of effector function is not HLA-restricted. This stimulation of PBMC with synthetic peptides represents an advance in current methodologies using tumor as the immunogen in that an unlimited supply of the synthetic immunogen can be obtained, whereas use of autologous tumor is limited to only the amount of tumor obtained.

Although the single mucin tandem repeat peptides are immunogenic, initial structure studies of the single tandem repeat peptide shows that there is little evidence for a preferred stable conformation. We have also proposed a model to describe the arrangement of the oligosaccharide side chains around the core protein. In the remaining two years of this work, we are progressing toward further describing the influence that the length of the core protein has on the structure and immunogenicity of this tumor-specific immunogen and refining the model of mucin.

To accomplish the aims of understanding the influence that protein length has on the structure and immunogenicity, we will continue to study synthetic and recombinant mucin peptides. Polypeptides of up to 60 amino acids (e.g. three tandem repeats of mucin) can be prepared by automated solid-phase protein synthesizers. However, the product yield decreases substantially with increasing length. Therefore, synthetic peptides of more than

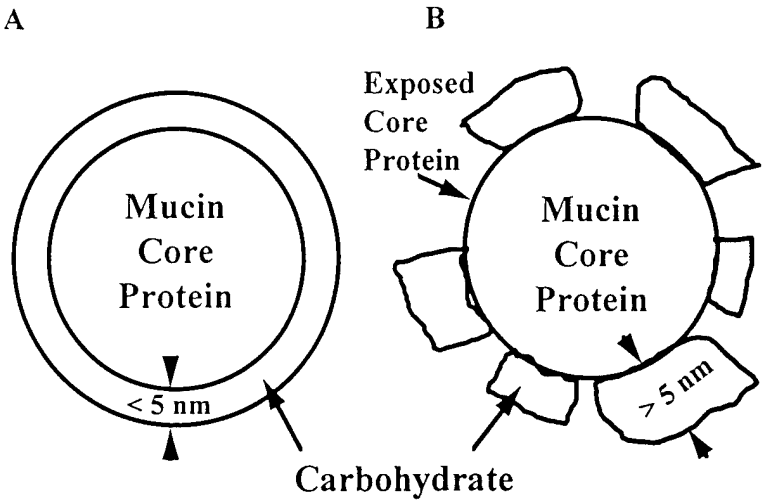

Figure 14. Possible models for the arrangement of the oligosaccharide side chains of human breast milk mucin along the core protein. (A) This model shows a uniformly thin coating of the core protein masking tumor-specific protein epitopes. (B) This model shows the exposed core protein that may contain non-tumor-specific epitopes, and tumor-specific epitopes masked by the normal glycosylation of this molecule.

two or three tandem repeats are not cost effective. Recombinant bacterial clones are a more effective approach to producing polypeptides containing more tandem repeats. A human mucin gene fragment consisting of seven tandem repeats in an *E. coli* expression system (21), but the protein product was subject to rapid degradation. We therefore decided to design a synthetic version of the mucin tandem repeat expressly for good protein production in *E. coli*. Furthermore, this recombinant gene will be designed for expression by fowlpox viruses. These viruses are capable of initiating infection of mammalian cells, but the infection is not productive, i.e., the virus does not replicate. Thus, fowlpox viruses have the potential of providing safe vaccines by producing antigens intracellularly (facilitating antigen presentation) when the inserted gene is designed to be expressed prior to replication.

In conclusion, the work on this grant is proceeding on schedule with regard to the specific aims of this grant. In the following years of this grant, the design and construction of additional mucin immunogens, both synthetic and recombinant proteins, will be pursued. This will further define structural features of tumor-specific mucin to better understand the immunogenicity of this molecule for use in the adoptive immunotherapy of breast and other adenocarcinomas and as potential vaccines against this disease. We look forward to the remaining years of this work to further our understanding of the structure-immunogenicity relationships of tumor-specific mucin immunogens for design of immunotherapies and vaccines against adenocarcinomas.

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conformation studies); Drs. David Millington and Robert Stevens (mass spectrometry); Dr. William Moddeman, Jannine Birkbeck and Bobby Russell (surface analysis); and Drs. Jane Lebkowski, Ramila Philip, Sohel Talib and Ji Yuan Wu (CTL expansion and characterization). This work was also supported, in part, by the Department of the Army Career Development Award #DAMD17-94-J-4161 (KED).

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Appendix.

Immunogenic Structural Features of a Breast Tumor-specific Epitope for Cancer Immunotherapy.

Grant No: DMAD17-94-J-4272

The following material is a listing of the publications, presentations and patent disclosures/applications that have resulted from funding from the above referenced Department of the Army grant during the current reporting period:

Publications:

1. Dombrowski, K.E., Wright, S.E., Birkbeck, J.C. & Moddeman, W.E. (1995) *X-ray Photoelectron Spectroscopy of Amino Acids, Polypeptides and Simple Carbohydrates* in Methods in Protein Structure Analysis (Atassi, M.Z. & Appella, E., eds) Plenum, NY, Ch. 22, 251-260.

Abstracts published:

1. A.A. Ribeiro, D.G. Davis, L.D. Spicer, S.E. Wright and K.E. Dombrowski (1996) *Gradient Assisted NOESY and ROESY for Mucin Peptide Conformational Studies in Water,* Poster #419, 37th Experimental NMR Conference, Asilomar, CA, March 17-22, 1996.
2. Ribeiro, A., Spicer, L.D., Wright, S.E. and Dombrowski, K.E. (1996) *NMR of Native and Mutant Peptides of Human Epithelial Mucin,* American Society for Biochemistry and Molecular Biology meeting, New Orleans, LA, June 2-6, 1996.
3. Dombrowski, K.E., Wright, S.E., Birkbeck, J.C., and Moddeman, W.E. (1996) *Surface Analysis of Proteins and Related Molecules by X-Ray Photoelectron Spectroscopy (XPS),* American Society for Biochemistry and Molecular Biology meeting, New Orleans, LA, June 2-6, 1996.
4. Wu, J.Y. and Wright, S.E. (1996) *Expansion of Cytolytic Effector Cells from a Breast Cancer Patient by Stimulation with Mucin Peptide and Antigen Presenting Cells,* American Association of Immunologists meeting, New Orleans, LA, June 2-6, 1996.

Presentations:

1. Wright, S.E., Lowe, K.E., Talib, S. Kilinski, L., Dombrowski, K.E., Lebkowski, J.S. and Philip, R. (1996) *Antigen-Specific Cytotoxic T-Lymphocyte (CTL) Response Induced by Tumor-specific MUC1 Mucin Peptide From Humans With Adenocarcinomas.* Presented at the 4th International Workshop on Carcinoma-Associated Mucins, Cambridge, U.K., July 27-31, 1996.

Patent Disclosure:

1. Dombrowski, K.E. and Moddeman, W.E. *Non-destructive Analysis of Proteins Bound to a Solid Support by X-ray Photoelectron Spectroscopy.*